EXHIBIT 6b

2. Human Epidemiological Evidence

The field of epidemiology plays a vital role in determining theteratogenicity of a pharmaceutical compound. The study of the occurrence of illness and disease in human populations, especially the cause(s) and determinants of patterns of disease, is a criticalcomponent to my work as a teratologist. Toxicological experiments are generally not conducted in pregnant women for obvious ethical reasons; therefore, teratologists often rely upon epidemiological studies of pregnancy outcomes related to maternal drug exposure to analyze human teratogenicity. Pertinent epidemiological studies, coupled with the animal experiments and application of Wilson's principles, inform any conclusion related to the teratogenicity of a medication. As such, teratologists must interpret and rely upon clinical case reports, drug pregnancy registries, prospective cohorts, retrospective cohorts, adverse event reporting, exposure monitoring systems, post-marketing surveillance, and epidemiological studies,including meta-analyses, to help inform their decisions concerning the teratogenic nature of a drug in the human situation. In assessing the weight-of-the-evidence for each epidemiological study, I employed these six Quality Assessment Points:

The Six Quality Assessment Points to be Evaluated in Human Studies:[18]

Human data	
Point to evaluate	**Areas of evaluation**
Study design or purpose	• Type of design • If study is appropriate for the question of interest • If question aims to generate versus test a hypothesis
Exposure classification	• If information is available about medication frequency, dose, and duration of use • If exposure is from an appropriate time window • If exposure is prospectively collected • If exposure is collected from an automated source • If exposure is validated
Outcome classification	• Outcome definition • Outcome ascertainment • Outcome diagnosis criteria • If outcome is validated
Control group	• If there is an internal control group • If control group is disease-based
Study size	• If power calculations are conducted • If confidence limits are provided • Potential for multiple testing
Confounding factors or biases	• If confounders controlled for • If additional biases accounted for and reduced

3. Systematic Reviews and Meta-Analyses

Hierarchy of Evidence, Level 1

These are comprehensive studies that combine and analyze the results of multiple research studies. In the Hierarchy of Evidence, systematic reviews and meta-analyses are at the top of the hierarchy. They are the highest levels of evidence because they provide a comprehensive and rigorous analysis of multiple studies on a particular topic.

A systematic review is a methodical way of examining all the scientific studies related to a specific topic or question. Researchers carefully select and analyze relevant studies that meet certain criteria to create an unbiased and complete summary of the evidence. The goal is to provide a clear answer to the research question based on all available information.

Within a systematic review, researchers often use a technique called meta-analysis. A meta-analysis combines the results from multiple studies, pooling their data together to obtain a more precise estimate of the treatment effect or association. This statistical approach allows researchers to analyze the combined evidence, providing a more comprehensive and reliable summary of the overall findings.

By combining a systematic review with a meta-analysis, researchers can evaluate the evidence more effectively. This helps them estimate the overall impact of a treatment or association more accurately and draw more reliable conclusions. Systematic reviews and meta-analyses are important tools used in evidence-based practice, decision-making, and shaping healthcare policies and guidelines.

Adverse Outcome Pathway: Framework for Analysis of Acetaminophen Exposures

Adverse Outcome Pathway (AOP) analysis is a framework used by various regulatory organizations to understand the causal relationship between chemical exposures and adverse effects on living organisms. AOPs are structured frameworks that provide a clear and transparent understanding of the biological events that lead to a specific adverse outcome. The process involves identifying key biological events, termed key events (KEs), and the relationships between them (links), leading to the adverse outcome (AO). By identifying the KEs involved in the pathway leading to AOs, AOPs provide a widely accepted foundation to evaluate the potential toxicity of chemicals. This approach is summarized in the AOP Fact Sheet (Figure 2).

Regulatory organizations use AOP analysis to support a variety of decision-making processes, including chemical prioritization, hazard identification, and risk assessment. The use of AOPs has become increasingly important as new chemicals and chemical mixtures are introduced into the environment. By providing a standardized framework for understanding the potential adverse outcomes associated with chemical exposures, AOPs support the development of effective risk management strategies that protect public health and the environment.

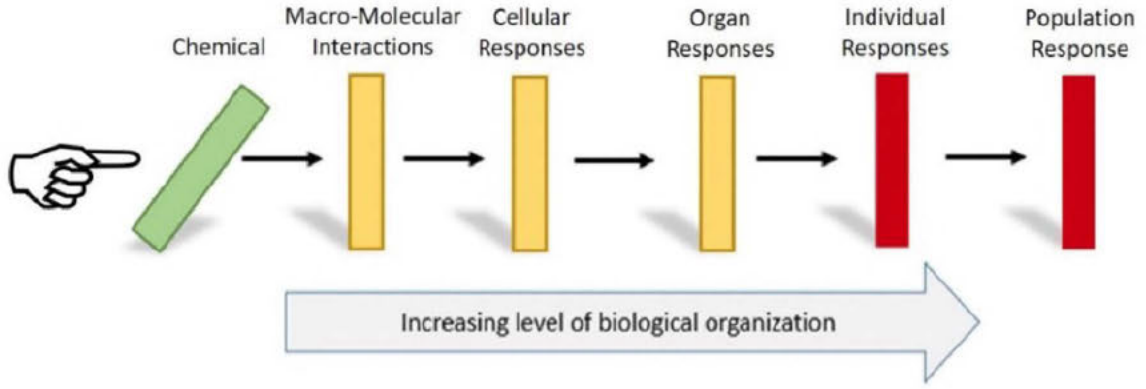

Figure 2. Adverse Outcome Pathway (AOP).[19] AOPs map how stressors interact within an organism to cause adverse outcomes.

[19] AOP Fact Sheet – US Environmental Protection Agency (US EPA).

BACKGROUND: ACETAMINOPHEN

Acetaminophen is an organic compound with a molecular formula of C8H9NO2 and a molecular weight of 151.16 g (CAS # 103-90-2). It is an acetyl derivative of p-aminophenol/4-aminophenol (Figure 3) and is also known as N-acetyl-p-aminophenol (APAP), paracetamol, or 4-acetamidophenol, and pharmaceutical brand names include Tylenol (J&J) and Panadol (GSK). While limited APAP synthesis and usage was reported in the 1800s, the Tylenol brand was first introduced and gained a wide market in 1955, by McNeil Laboratories, as a pain-relief/analgesic and fever-reducing/anti-pyretic medication for children. Johnson & Johnson acquired McNeil Laboratories in 1959 and began marketing Tylenol over the counter (OTC) and towards adults by 1961. Marketed as a safe alternative to aspirin, Johnson & Johnson reports Tylenol as one of the company's most widely used and iconic products.[20]

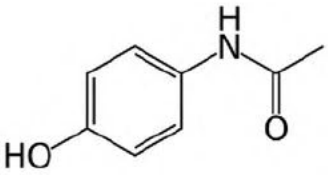

Figure 3. Chemical Structure of Acetaminophen (APAP)

The Aniline Analgesics

Acetanilide, phenacetin, and paracetamol (acetaminophen, APAP) are the original members of the drug class referred to as aniline analgesics. Acetanilide was the first of these molecules to gain usage as an antipyretic, when it was reported in 1886 that it was mistakenly used in place of naphthalene to treat intestinal worms and resulted in a reduction in fever.[21] The use of acetanilide was limited due to toxicity resulting in methemoglobinemia and cyanosis. As the use of acetanilide was limited by toxicity, the search for other aniline derivatives resulted in the introduction of phenacetin and paracetamol. Paracetamol was initially overlooked due to an assumption that phenacetin was less toxic, so phenacetin was the predominate aniline analgesic. Phenacetin usage continued for several decades, but usage gradually fell after it was reported in 1948, that metabolites of acetanilide included aniline and paracetamol.[22] Phenacetin was ultimately withdrawn from the market in the 1970s and 1980s, with reports of a high potential for misuse and an unfavorable benefit-to-risk ratio.[23] There were also reports in the 1960s, that phenacetin was associated with renal lesions and nephrotoxicity.[24] Phenacetin was eventually classified by the International Agency for Research on Cancer (IARC) in 1987 as probably carcinogenic to humans (Group 2A) and analgesic mixtures containing phenacetin as carcinogenic (Group 1).[25] While APAP is promoted as one of the most popular and safe pain-relief medications, **APAP toxicity is responsible for almost**

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[20] Company History, Johnson & Johnson, https://ourstory.jnj.com/timeline

[21] Brune et al. Acetaminophen/paracetamol: A history of errors, failures and false decisions. Eur J Pain. 2015 Aug;19(7):953-65. doi: 10.1002/ejp.621. Epub 2014 Nov 27. PMID: 25429980.

[22] Brodie and Axlerod. The estimation of acetanilide and its metabolic products, aniline, N-acetyl p-aminophenol and p-aminophenol, free and total conjugated, in biological fluids and tissues. J Pharmacol Exp Ther. 1948 Sep;94(1):22-8. PMID: 18885610.

[23] FDA, Federal Register, Vol 48, No 194, Wednesday, October 5, 1983.

[24] Tan et al. Is phenacetin a nephrotoxin? a report on twenty-three users of the drug. Calif Med. 1964 Aug;101(2):73-7. PMID: 14180501; PMCID: PMC1515485.

[25] IARC: Overall evaluations of carcinogenicity: an updating of IARC monographs volumes 1 to 42. 1987, Lyon: International Agency for Research on Cancer, 7: https://monographs.iarc.who.int/wp-content/uploads/2018/06/Suppl7-121.pdf

half (46%) of all acute liver failure cases in the United States, and it is estimated that half of these cases are unintentional.[26,27]

1. Acetaminophen and Aniline

Acetaminophen remains as the only aniline analgesic still in use today, and as explained below, because APAP is a metabolite of aniline, the wide-spread use of industrial aniline in the environment results in nearly universal human exposure to APAP, although at very low levels.

The other members of the aniline analgesic class have been banned for clinical use, but aniline remains an important source material in various chemical industries (e.g., rubber, pesticides, and pharmaceuticals). An IMARC market overview indicates the global aniline market size reached 9.4 million tons in 2022.[28] As indicated below, industrialized population are reported to be ubiquitously exposed to aniline, and thereby APAP as a metabolite of aniline.

A European Chemicals Bureau risk assessment report has estimated that the human exposure to aniline from fruit and vegetables is approximately 0.11 mg/kg bodyweight per day, while humans near industrial sources have an exposure burden estimated at 0.74 mg/kg/day.[29] Biological monitoring in Germany reported ubiquitous urinary excretion of APAP in the general population and examined possible sources: (1) direct intake of paracetamol through medication, (2) paracetamol residues in the food chain and (3) environmental exposure to aniline or related substances that are metabolized into APAP.[30] The proposed conversion of aniline to APAP was tested in an animal model, and aniline was shown to be converted nearly completely in the liver of the mouse to APAP.[31] Aniline conversion to APAP was then tested in four healthy male volunteers with a 5 mg dose of isotope-labeled aniline, and APAP was the predominant urinary aniline metabolite representing 55.7-68.9 % of the oral dose.[32]

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[26] Ramachandran A, Jaeschke H. Acetaminophen Hepatotoxicity. Semin Liver Dis. 2019 May;39(2):221-234. doi: 10.1055/s-0039-1679919. Epub 2019 Mar 8. PMID: 30849782; PMCID: PMC6800176.

[27] Agrawal and Khazaeni. Acetaminophen Toxicity. [Updated 2023 Feb 12]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK441917/

[28] Aniline Market Size, Share, Price Trends, Analysis, 2023-2028 (imarcgroup.com): https://www.imarcgroup.com/aniline-market

[29] EU Summary risk assessment report (europa.eu): https://echa.europa.eu/documents/10162/462b7066-c639-4883-b384-3daf4ec88ded

[30] Dierkes G, et al. N-Acetyl-4-aminophenol (paracetamol), N-acetyl-2-aminophenol and acetanilide in urine samples from the general population, individuals exposed to aniline and paracetamol users. Int J Hyg Environ Health. 2014 Apr-May;217(4-5):592-9. doi: 10.1016/j.ijheh.2013.11.005. Epub 2013 Dec 6. PMID: 24370547.

[31] Holm JB, et al. Aniline Is Rapidly Converted Into Paracetamol Impairing Male Reproductive Development. Toxicol Sci. 2015 Nov;148(1):288-98. doi: 10.1093/toxsci/kfv179. Epub 2015 Aug 10. PMID: 26259604.

[32] Modick H, et al. Human metabolism and excretion kinetics of aniline after a single oral dose. Arch Toxicol. 2016 Jun;90(6):1325-33. doi: 10.1007/s00204-015-1566-x. Epub 2015 Aug 2. PMID: 26233686.

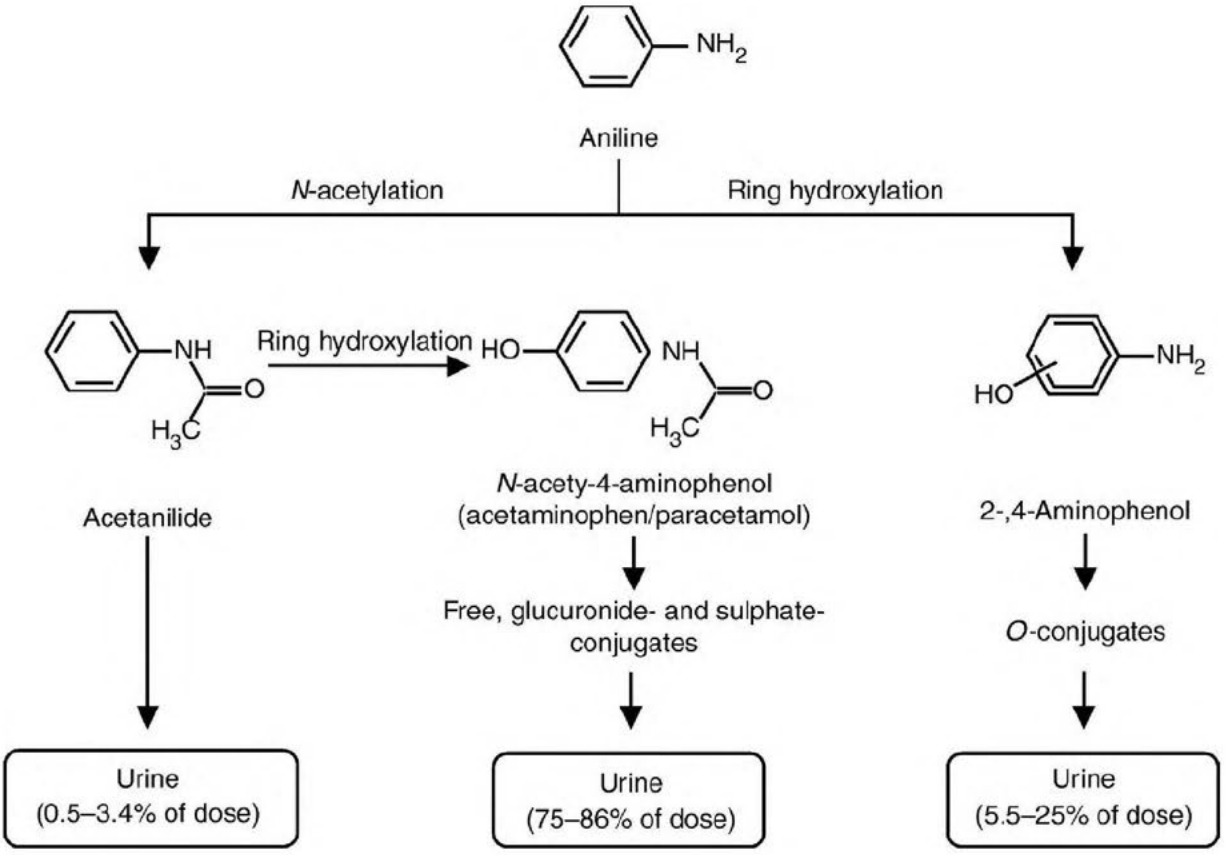

Figure 4. Simplified Metabolism of Aniline to Acetaminophen (APAP).[33]

Ubiquitous environmental exposures to the industrial chemical aniline results in conversion to APAP in the liver of laboratory animals and humans, resulting in a low-level chronic exposure to and excretion of APAP (Figure 4). The pharmaceutical consumption of APAP increases this exposure above background level, producing environmental (background) versus pharmaceutical exposures that differ by 1000-fold (Figure 5).

[33] Modick H, et al. Ubiquitous presence of paracetamol in human urine: sources and implications. Reproduction. 2014 Mar 4;147(4):R105-17. doi: 10.1530/REP-13-0527. PMID: 24451225.

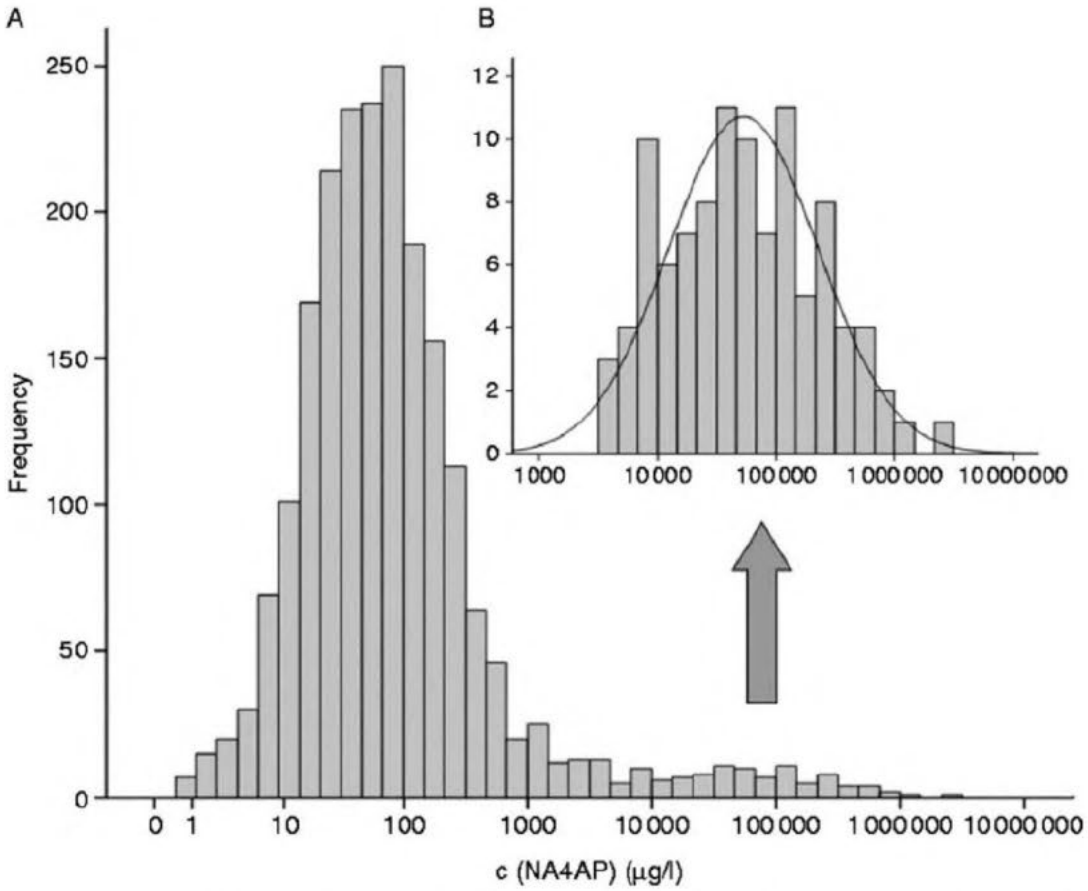

Figure 5. Distribution of Urinary APAP. Concentrations in random spot urine samples obtained from the general population: (A) all samples (n=2098) and (B) samples of the high-exposure group (n=106; c>4000µg/l). The high exposures are due to pharmacological intake of APAP. Line indicates normal distribution curve.[34]

This study showed that the general population is exposed to APAP at a relatively low level, with a median exposure of about 80-100 micrograms/L, and this exposure is proposed to originate from the metabolism of the industrial chemical aniline into APAP. These low concentrations contrast with the concentrations found in people reported to have recently taken APAP as a pharmaceutical (Figure 5, B). Pharmaceutical APAP intake results in increased urinary APAP to approximately 1,000 times greater than the general population. Accordingly, APAP is expected to be detected in the general population, even if this population has not taken or reported recent use of APAP, and this background level of APAP is reported to be due to environmental exposure to aniline and conversion of it into APAP. **This review of aniline conversion into APAP is provided to explain the reported detection of APAP in human biologics, even in the absence of reported pharmaceutical intake of APAP.**

Acetaminophen Metabolism

Acetaminophen is a weak organic acid with moderate lipid-solubility that allows passive diffusion across the small intestine and cellular membranes. The absorption from the gastrointestinal tract is dose-

[34] Modick H, et al. Ubiquitous presence of paracetamol in human urine: sources and implications. Reproduction. 2014 Mar 4;147(4):R105-17. doi: 10.1530/REP-13-0527. PMID: 24451225.

dependent, and ranges from 70-90%.[35] The Cmax was reported between 30-90 minutes when given to 144 subjects, with a mean concentration of 16 microgram/mL in whole blood when subjects were given a dose of 1000mg.[36] Once absorbed, the metabolism of acetaminophen has been well documented for decades (Figure 6). Briefly, about 50-70% and 25-35% of a therapeutic dose is metabolized and excreted in the urine as glucuronic and sulfuric acid conjugates, respectively, whereas the toxic-reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), gives rise to mercapturic acid and cysteine conjugates and accounts for 5-15% of the dose.[37]

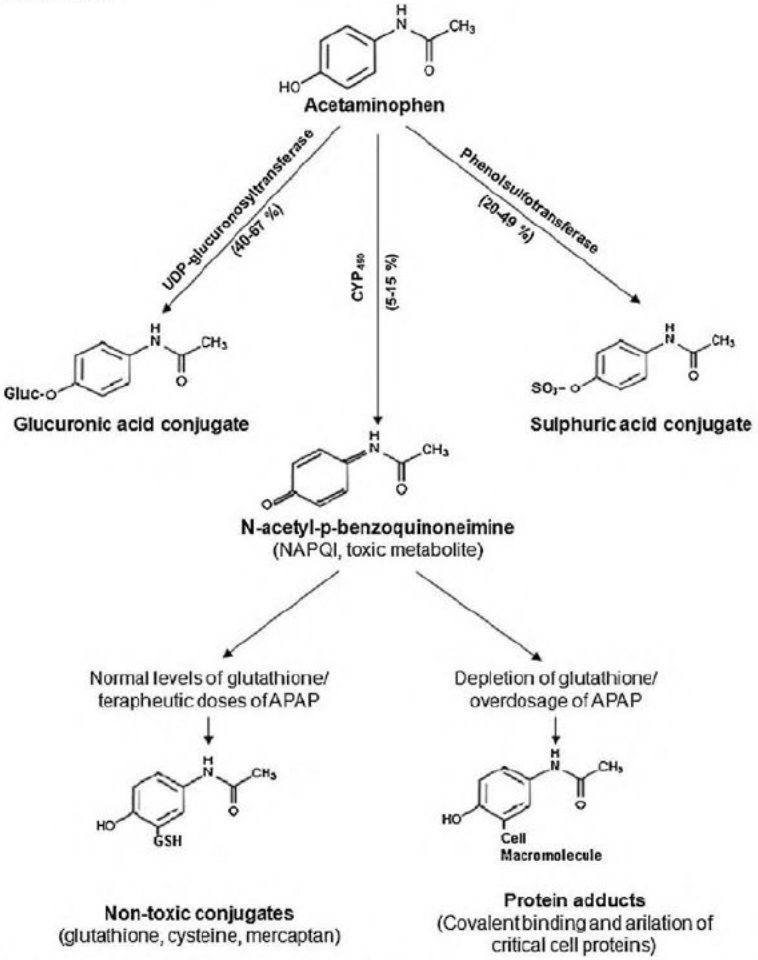

Figure 6. Liver Acetaminophen (APAP) Metabolism. There are three main metabolic pathways for APAP in liver after administration of therapeutic or toxic doses.[38]

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[35] Forrest et al. Clinical pharmacokinetics of paracetamol. Clin Pharmacokinet. 1982 Mar-Apr;7(2):93-107. doi: 10.2165/00003088-198207020-00001. PMID: 7039926.

[36] Gwilt et al. The absorption characteristics of paracetamol tablets in man. J Pharm Pharmacol. 1963 Jul;15:445-53. doi: 10.1111/j.2042-7158.1963.tb12812.x. PMID: 13951544.

[37] Prescott LF. Kinetics and metabolism of paracetamol and phenacetin. Br J Clin Pharmacol. 1980 Oct;10 Suppl 2(Suppl 2):291S-298S. doi: 10.1111/j.1365-2125.1980.tb01812.x. PMID: 7002186; PMCID: PMC1430174.

[38] Ghanem et al. Acetaminophen from liver to brain: New insights into drug pharmacological action and toxicity. Pharmacol Res. 2016 Jul;109:119-31. doi: 10.1016/j.phrs.2016.02.020. Epub 2016 Feb 26. PMID: 26921661; PMCID: PMC4912877.

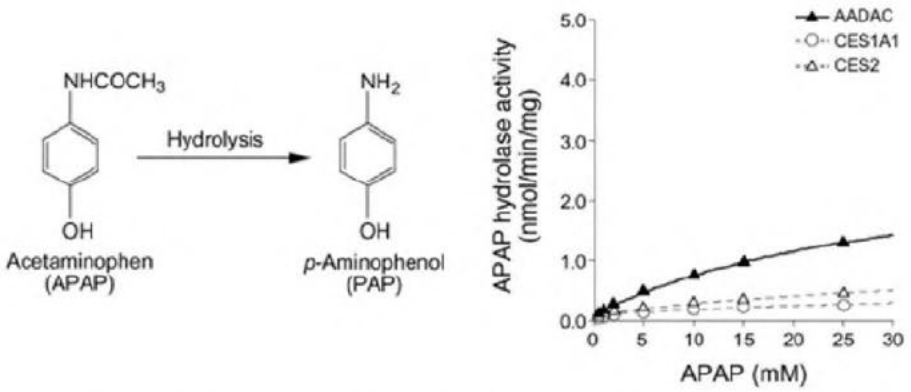

Figure 7. Acetaminophen (APAP) Hydrolysis. (Left) The hydrolysis of APAP into PAP. (Right) The relative activity of carboxylesterases, CES1A1 and CES2, and arylacetamide deacetylase (AADAC) on APAP hydrolysis were reported in 2010.[39]

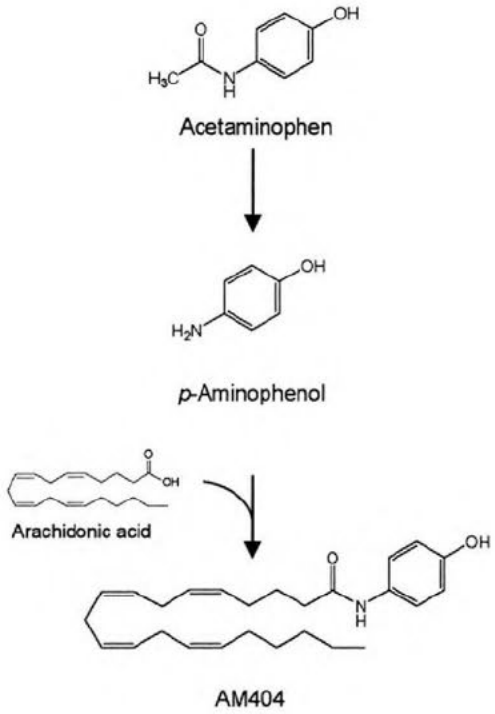

Figure 8. Acetaminophen (APAP) Hydrolysis, Arachidonic Acid Conjugation, and AM404.[40]

The dependence of AM404 formation from PAP by fatty acid amide hydrolase (FAAH) was reported in 2005.[40] APAP can also be metabolized via hydrolysis by AADAC (Figure 7). This process is the first step in arachidonic acid conjugation of PAP by FAAH, forming AM404 (Figure 8). FAAH is found in various

[39] Watanabe et al. Arylacetamide deacetylase is a determinant enzyme for the difference in hydrolase activities of phenacetin and acetaminophen. Drug Metab Dispos. 2010 Sep;38(9):1532-7. doi: 10.1124/dmd.110.033720. Epub 2010 Jun 11. PMID: 20542992.

[40] Högestätt et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem. 2005 Sep 9;280(36):31405-12. doi: 10.1074/jbc.M501489200. Epub 2005 Jun 29. PMID: 15987694.

tissues, including liver, thyroid, testes, and brain, with high expression in the dentate gyrus, hippocampus, orbital cortex, primary motor and primary somatosensory cortex.[41]

Pharmaceutical Mechanisms of Action for Acetaminophen

The indications of acetaminophen include headache, pain, and fever. The suggested therapeutic dose of acetaminophen for adults is two tablets of 325mg, 500 mg, or 650mg, taken orally every 4-8 hours, with a maximum of 4 grams for any 24-hour period. Despite decades of use, the mechanism of action for acetaminophen has not been precisely quantified. There are currently multiple mechanisms of action proposed and numerous questions remain regarding the relative impact of each proposed mechanism on biological impacts (Figure 9).

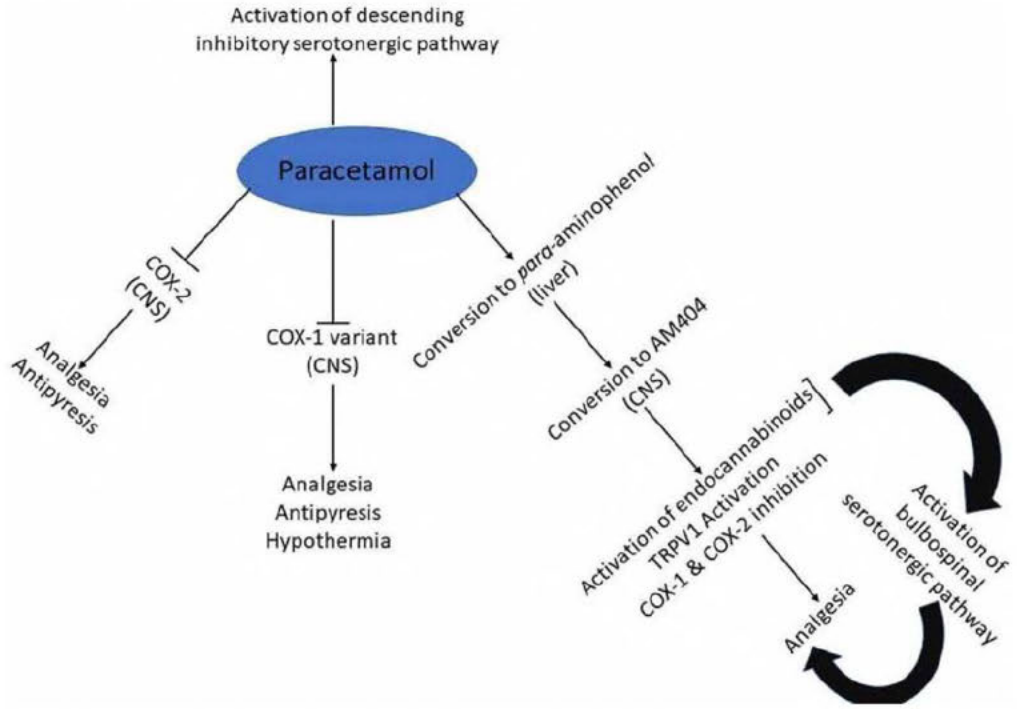

Figure 9. Schematic Representation of Proposed Pharmacological Mechanisms of Action of Paracetamol.[42]

1. COX Interactions

The proposed mechanisms of actions are based on activities in the central nervous system (CNS), where APAP is reported to increase pain threshold and thereby cause pain relief. One of the first proposed mechanisms of the analgesic action of APAP was the inhibition of cyclooxygenase enzymes (COX-1 and

[41] Egertováet al. Comparative analysis of fatty acid amide hydrolase and cb(1) cannabinoid receptor expression in the mouse brain: evidence of a widespread role for fatty acid amide hydrolase in regulation of endocannabinoid signaling. Neuroscience. 2003;119(2):481-96. doi: 10.1016/s0306-4522(03)00145-3. PMID: 12770562.

[42] Ayoub SS. Paracetamol (acetaminophen): A familiar drug with an unexplained mechanism of action. Temperature (Austin). 2021 Mar 16;8(4):351-371. doi: 10.1080/23328940.2021.1886392. PMID: 34901318; PMCID: PMC8654482.

COX-2), which are involved in prostaglandin (PG) synthesis.[43,44] Additional studies have also introduced the potential of inhibition of a splice variant of COX-1, referred to as COX-1b or COX-3 in various publication.[45,46]

2. Serotonergic Interactions

In addition to COX inhibition, activation of the descending inhibitory serotonergic pathway by APAP has also been proposed to provide analgesic action in animal models and human. The serotonergic receptors implicated in these studies include 5-HT1A,[47] 5-HT3,[48] and 5-HT7.[49] In addition, depletion of serotonin (5-HT), by the administration of p-chlorophenylalanine, was shown to attenuate the analgesic action of APAP,[50] but the influence of APAP on serotonergic levels and signaling is proposed to be indirect (Figure 10), as APAP has not shown a relevant affinity for any of the serotonin receptors or transporter.[51]

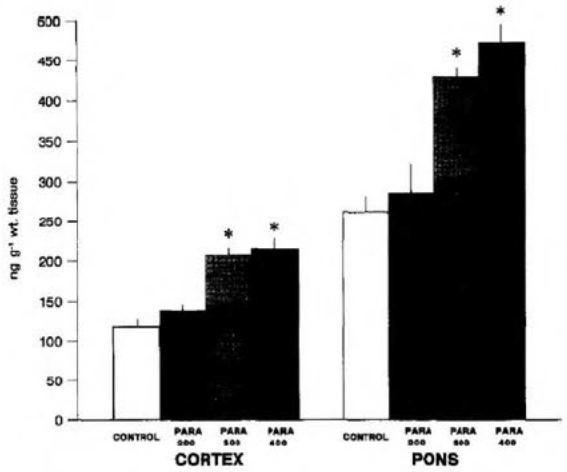

Figure 10. Impact of Acetaminophen (APAP/PARA) at 200, 300, and 400mg/Kg on Serotonin (5-HT ng/g tissue) in Cortical (CORTEX) and Pontine (PONS) Brain Areas. Values are average +/- SEM for N=6 rats per group, *

[43] Flower and Vane. Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). Nature. 1972 Dec 15;240(5381):410-1. doi: 10.1038/240410a0. PMID: 4564318.
[44] Mitchell et al. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proc Natl Acad Sci U S A. 1993 Dec 15;90(24):11693-7. doi: 10.1073/pnas.90.24.11693. PMID: 8265610; PMCID: PMC48050.
[45] Willoughby, et al. COX-1, COX-2, and COX-3 and the future treatment of chronic inflammatory disease. Lancet. 2000 Feb 19;355(9204):646-8. doi: 10.1016/S0140-6736(99)12031-2. PMID: 10696997.
[46] Chandrasekharan et al. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. Proc Natl Acad Sci U S A. 2002 Oct 15;99(21):13926-31. doi: 10.1073/pnas.162468699. Epub 2002 Sep 19. PMID: 12242329; PMCID: PMC129799.
[47] Karandikar et al. Effect of drugs modulating serotonergic system on the analgesic action of paracetamol in mice. Indian J Pharmacol. 2016 May-Jun;48(3):281-5. doi: 10.4103/0253-7613.182874. PMID: 27298498; PMCID: PMC4900001.
[48] Pelissier et al. Paracetamol exerts a spinal antinociceptive effect involving an indirect interaction with 5-hydroxytryptamine3 receptors: in vivo and in vitro evidence. J Pharmacol Exp Ther. 1996 Jul;278(1):8-14. PMID: 8764329.
[49] Dogrul et al. Systemic paracetamol-induced analgesic and antihyperalgesic effects through activation of descending serotonergic pathways involving spinal 5-HT_7 receptors. Eur J Pharmacol. 2012 Feb 29;677(1-3):93-101. doi: 10.1016/j.ejphar.2011.12.016. Epub 2011 Dec 21. PMID: 22206817.
[50] Pini et al. The antinociceptive action of paracetamol is associated with changes in the serotonergic system in the rat brain. Eur J Pharmacol. 1996 Jul 11;308(1):31-40. doi: 10.1016/0014-2999(96)00261-0. PMID: 8836629.
[51] Raffa and Codd. Lack of binding of acetaminophen to 5-HT receptor or uptake sites (or eleven other binding/uptake assays). Life Sci. 1996;59(2):PL37-40. doi: 10.1016/0024-3205(96)00273-1. PMID: 8699917.

$P < 0.05$ vs. control values (ANOVA followed by Student-Newman-Keuls' test).[52] This 5-HT response by APAP has also been reported in the frontal cortex and brainstem.[53]

While APAP has not been shown to have a high affinity for serotonin receptors or the serotonin transporter (SERT), the activity of APAP on pain (antinociceptive) has been shown to be dependent in part on the ability of APAP to modify serotonin concentrations. These indirect serotonergic interactions have been reported in multiple studies. Regarding direct interactions, at 10 μM, APAP inhibited less than 10% of radioligand binding to serotonin receptors or transporter.[51] Regarding indirect interactions, the involvement of serotonin receptors, 5-HT(1A/B), in the antinociceptive effect of APAP was demonstrated by modifying the interactions of known antagonists of 5-HT1A and 5-HT1B in mice.[54] Similar results have also been reported in rats, indicating that APAP modifies the interaction between serotonin and the 5-HT1B receptor.[55,56] Loss of 5-HT1B is reported to alter behavior in mice including altered impulse control, behavioral disinhibition, and stimulus specific hyperreactivity.[57,58] The ability of APAP to modify serotonin and interactions with 5-HT1B has CNS and vascular impacts. For example, 5-HT1b also mediates vasoconstriction in human umbilical arteries.[59,60,61]

The influence of serotonin on vascular function, and specifically the biphasic dose response of 5-HT on human umbilical arteries has been observed in numerous studies.[62,63,64] Moreover, the naming and function of serotonin is actually based on its activity as a serum vasoconstrictor.[65] Excess 5-HT is also a demonstrated teratogen in various animal models, capable of producing a wide-spectrum of congenital

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[52] Pini et al. The antinociceptive action of paracetamol is associated with changes in the serotonergic system in the rat brain. Eur J Pharmacol. 1996 Jul 11;308(1):31-40. doi: 10.1016/0014-2999(96)00261-0. PMID: 8836629.

[53] Vijayakaran K, et al. Arsenic decreases antinociceptive activity of paracetamol: possible involvement of serotonergic and endocannabinoid receptors. Environ Toxicol Pharmacol. 2014 Sep;38(2):397-405. doi: 10.1016/j.etap.2014.07.001. Epub 2014 Jul 10. PMID: 25128769.

[54] Roca-Vinardell et al. The role of 5-HT1A/B autoreceptors in the antinociceptive effect of systemic administration of acetaminophen. Anesthesiology. 2003 Mar;98(3):741-7. doi: 10.1097/00000542-200303000-00025. PMID: 12606921.

[55] Courade et al. 5-HT receptor subtypes involved in the spinal antinociceptive effect of acetaminophen in rats. Eur J Pharmacol. 2001 Nov 30;432(1):1-7. doi: 10.1016/s0014-2999(01)01464-9. PMID: 11734181.

[56] Sandrini et al. Differential involvement of central 5-HT1B and 5-HT3 receptor subtypes in the antinociceptive effect of paracetamol. Inflamm Res. 2003 Aug;52(8):347-52. doi: 10.1007/s00011-003-1185-5. PMID: 14504673.

[57] Ramboz et al. 5-HT1B receptor knock out--behavioral consequences. Behav Brain Res. 1996;73(1-2):305-12. doi: 10.1016/0166-4328(96)00119-2. PMID: 8788525.

[58] Bouwknecht et al. Absence of 5-HT(1B) receptors is associated with impaired impulse control in male 5-HT(1B) knockout mice. Biol Psychiatry. 2001 Apr 1;49(7):557-68. doi: 10.1016/s0006-3223(00)01018-0. PMID: 11297712.

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[60] Gupta et al. Functional reactivity of 5-HT receptors in human umbilical cord and maternal subcutaneous fat arteries after normotensive or pre-eclamptic pregnancy. J Hypertens. 2006 Jul;24(7):1345-53. doi: 10.1097/01.hjh.0000234115.40648.88. PMID: 16794484.

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malformations.[66,67,68] While APAP is reported to increase serotonin in the CNS, APAP is reported to inhibit serotonin release by platelets and reduce vasoconstriction.[69,70] The proposed mechanism for these divergent interactions is via differences in COX enzymes, as indicated above. Decreased plasma 5-HT due to pharmacological or genetic depletion has also been reported to increase the hepatotoxicity of APAP in mice.[71]

3. Cannabinoid Interactions

While the main metabolic routes for APAP in the liver have been studied for decades, in 2005 another APAP metabolite, p-aminophenol (PAP), was identified in the liver, blood, and brain, accounting for 1-2% of APAP metabolism,[72] and which is further conjugated with arachidonic acid to form the bioactive fatty acid amide N-arachidonoyl phenolamine (AM404) in the brain.[73] The conversion of PAP to AM404 was reported to be dependent on fatty acid amide hydrolase (FAAH), as mutant mice lacking FAAH did not produce the AM404 metabolite. AM404 was also reported to be a potent activator (nM) of TRPV1, a ligand at cannabinoid CB1 receptors and an inhibitor of cellular anandamide uptake, leading to increased levels of anandamide, an endogenous cannabinoid. Additional studies have reported that AM404 is also an inhibitor of the arachidonoylethanolamide (AEA) membrane transporter (AMT) and can activate the vanilloid (capsaicin) receptor (VR1) in the nM range.[74]

Anandamide is an endogenous modifier of cannabinoid signaling via cannabinoid CB1 receptor, and activation by endogenous or exogenous cannabinoids can produce a range of different impacts on learning, behavior, and development. In order to examine the role of loss of CB1, mutant mice were produced that had CB1 mutated. These animals displayed disruption of learning and behavior, specifically the animals were unable to unlearn bad memories, termed extinction of aversive memories.[75] This is based on laboratory testing of fear conditioning, such that a ringing tone or flash of light is coupled with pain, via a shock or other noxious exposure. After a few times, the animal learns to respond fearfully to the ringing tone or light. Extinction can then be performed by allowing the animal to hear the sound or see the light

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[75] Marsicano et al. The endogenous cannabinoid system controls extinction of aversive memories. Nature. 2002 Aug 1;418(6897):530-4. doi: 10.1038/nature00839. PMID: 12152079.

without anything bad happening, so they eventually learn to dissociate the tone or light from a bad experience, resulting in extinction of the aversive memory. Marsicano et al. tested CB1 mutant (-/-) and wildtype (+/+) by exposing them to a sound, then getting a small electric shock to their feet.[75] After the mice learned this association, they froze or stopped moving when they heard the sound, a sign of fear or heightened awareness. The researchers found both groups of mice learned the association between the sound and the shock equally well. However, when the researchers played the sound without giving the electric shock, the wildtype mice gradually stopped freezing, while the CB1(-/-) mice continued to freeze. These data indicate that CB1 and cannabinoid signaling is important for the process of forgetting/extinguishing fearful/aversive memories.

The impact of cannabinoids has also been shown to have regional or cell type specific effects. Using conditional mutant mice allows genetic mutations to be produced or coupled to the expression of a specific gene which may only be expressed in a specific region of the brain or body. In a study by Lafenêtre et al., mutant mice were produced that lacked CB1 in the cortical glutamatergic neurons or GABAergic neurons.[76] Testing utilized novel palatable food or a novel object and the responses to these stimuli were analyzed in mutant and wildtype mice over several days. The results of this study indicate that cannabinoid signaling can exert region-specific functions and balances between novelty seeking and behavioral inhibition. Specifically, loss of CB1 receptors expressed in cortical glutamatergic neurons produced a delay or latency to approach and eat novel food, and loss of CB1 receptor in inhibitory GABAergic neurons resulted in increased palatable food consumption, similar interactions with novel objects, and increasing novelty seeking behaviors. The authors described the importance of this work in relation to ADHD,

> The balance between novelty seeking and safety assessment is a key feature of adaptive behavior, and alterations in this equilibrium can lead to neuropsychiatric disorders. Excessive novelty seeking is a main form of pathological impulsivity, which is among the symptoms that define attention deficit hyperactivity disorder (ADHD).

Based on these studies, a variety of learning and adaptive behaviors can also be influenced by cannabinoid signaling. As indicated below, APAP can also influence similar testing and behavioral outcomes in animal models with pre, peri, or postnatal exposures. Regarding molecular interactions with APAP and its metabolites, the influence is diverse and includes COX inhibition, and the serotonergic, vanilloid, and cannabinoid signaling pathways are also part of the pharmacological mechanisms of action.[77]

Acetaminophen Toxicity

The Merck Manual reports the following summary for oral route APAP exposure,

> Immediate-release acetaminophen is rapidly and almost completely absorbed from the gastrointestinal (GI) tract, primarily the small intestine. Bioavailability ranges from 85% to 98%. Peak plasma concentrations occur within 30 to 60 minutes and range from 7.7 to 17.6 mcg/mL

[76] Lafenêtre et al. Bidirectional regulation of novelty-induced behavioral inhibition by the endocannabinoid system. Neuropharmacology. 2009 Dec;57(7-8):715-21. doi: 10.1016/j.neuropharm.2009.07.014. Epub 2009 Jul 14. PMID: 19607846. and

[77] Ohashi and Kohno. Analgesic Effect of Acetaminophen: A Review of Known and Novel Mechanisms of Action. Front Pharmacol. 2020 Nov 30;11:580289. doi: 10.3389/fphar.2020.580289. PMID: 33328986; PMCID: PMC7734311.

> after a single 1,000 mg dose and 7.9 to 27 mcg/mL at steady state after 1,000 mg every 6 hours in adult patients….

This estimate is based on a single therapeutic dose (1000mg), which is a human reference dose of 16.7mg/Kg, based on a 60Kg reference human, and

> To cause toxicity, an acute oral overdose must total ≥ 150 mg/kg (about 7.5 g in adults) within 24 hours.[78]

The Merck Manual reports an acute toxic dose is 7.5 grams over 24hrs, which is less than twice the maximum recommended dose of 4 grams (reference human dose of 66.6mg/Kg/day) over 24 hours. It is also noted that aspartate aminotransferase (AST) and alanine aminotransferase (ALT), being liver enzymes, are reported to correlate with the stage of poisoning,

> "Low-level transaminase elevations (e.g., up to 2 or 3 times the upper limit of normal) may occur in adults taking therapeutic doses of acetaminophen for days or weeks. These elevations appear to be transient, usually resolve or decrease within a few days (even with continued acetaminophen use), are usually clinically asymptomatic, and are probably insignificant."

Regarding liver enzymes,

> "If AST and ALT levels are normal (< 50 IU/L [0.83 microkat/L]), and the acetaminophen level is < 10 mcg/mL (< 66 micromol/L), significant hepatotoxicity is very unlikely.
>
> If AST and ALT levels are normal but the acetaminophen level is ≥ 10 mcg/mL (> 66 micromol/L), significant hepatotoxicity is possible; AST and ALT levels are remeasured after 24 hours. If repeat AST and ALT levels are normal, significant hepatotoxicity is unlikely; if the levels are high, significant hepatotoxicity is assumed.
>
> If initial AST and ALT levels are high, regardless of the acetaminophen level, significant hepatotoxicity is assumed."

These reference summaries on therapeutic and toxic dosages, metabolism, and resulting blood concentrations are important to consider regarding dosages with reported human toxicity in the literature and dosages used in animal model toxicity studies, reviewed below.

In regard to AST and ALT concentrations with APAP overdose in humans, initial clinical reports indicated there were elevations in liver enzymes, increased serum insulin, and altered glucose and lactate metabolism.[79,80] Overdosing estimates ranged from 12-50 grams, and the resulting AST ranged from 236 to >5000 IU/L. By the early 1970s, reported hepatotoxicity was recognized as the major manifestation of

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[78] Acetaminophen Poisoning (N-acetyl-para-aminophenol; APAP). By Gerald F. O'Malley , DO, Grand Strand Regional Medical Center; Rika O'Malley , MD, Grand Strand Medical Center. Last review/revision Jun 2022 | Modified Sep 2022

[79] Record et al. Disturbances in glucose metabolism in patients with liver damage due to paracetamol overdose. Clin Sci Mol Med. 1975 Nov;49(5):473-9. doi: 10.1042/cs0490473. PMID: 1192705.

[80] Record et al. Disturbances of lactate metabolism in patients with liver damage due to paracetamol overdose. Metabolism. 1981 Jul;30(7):638-43. doi: 10.1016/0026-0495(81)90076-7. PMID: 7242370.

APAP overdose in experimental animals and humans. The treatment for APAP overdose or poisoning is currently N-acetylcysteine (NAC). This remedy was initially reported and established in mice. Briefly, a lethal dose caused death in ~80% of mice (1200mg/Kg, ~LD80), but treatment with NAC at an equal mass dose (1200mg/Kg) within a few hours of APAP exposure (<4.5hrs) resulted in 85-100% rescue.[81] The following year, 1976, a clinical study reported the utility of NAC in preventing liver damage caused by APAP overdose if treatment was started within 10hrs of APAP exposure.[82] In patients starting treatment after 10hrs, liver damage was reported and indicated by AST or ALT above 1,000 IU/L, with all of these patients reaching 10,000 IU/L for AST or ALT.

Human Equivalent Dosages

To compare the effects of chemical exposures between humans and animal models, dosages are adjusted based on established metabolic differences. Metabolism is generally faster in smaller animals, so animal dosages (mg/Kg) require adjustment to produce comparable human dosages (mg/Kg). For example, a study can administer 50mg of a drug per kilogram of bodyweight (50mg/Kg). While this dosage could be used on humans or animals directly, adjustments for bodyweight (in Kg) are not sufficient to account for differences in metabolism. As indicated above, toxicity risk increases with APAP human dosages at 150 mg/kg, while it takes ~950-1200 mg/kg (PO) to produce a lethal dose to 50% (LD_{50}) of mice.[83] **Animal-human dosages (mg/Kg) should not be directly compared, because differences in size and metabolism necessitate a conversion.** These conversions produce Human Equivalent Dosages (HED) from animal dosages. The U.S. Department of Health and Human Services Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER) has provided Guidelines for Industry covering this topic, see Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers (Table 1).[84] As indicated below, a dose in mice (mg/Kg) can be converted to a HED by dividing it by 12.3, and a mg/Kg dose in rats can be converted to a HED by dividing by 6.2.

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[81] Piperno and Berssenbruegge. Reversal of experimental paracetamol toxicosis with N-acetylcysteine. Lancet. 1976 Oct 2;2(7988):738-9. doi: 10.1016/s0140-6736(76)90030-1. PMID: 61415.

[82] Prescott et al. Treatment of paracetamol (acetaminophen) poisoning with N-acetylcysteine. Lancet. 1977 Aug 27;2(8035):432-4. doi: 10.1016/s0140-6736(77)90612-2. PMID: 70646.

[83] Guasch et al. Pharmaco-toxicological effects of acetaminophen in rodents. Battery of tests to screen potential analgesic acetaminophen derivatives. Methods Find Exp Clin Pharmacol. 1990 Mar;12(2):141-8. PMID: 2319838.

[84] Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers, JULY 2005: https://www.fda.gov/media/72309/download

Table 1

Table 1: Conversion of Animal Doses to Human Equivalent Doses Based on Body Surface Area

Species	To Convert Animal Dose in mg/kg to Dose in mg/m², Multiply by k_m	To Convert Animal Dose in mg/kg to HED[a] in mg/kg, Either:	
		Divide Animal Dose By	Multiply Animal Dose By
Human	37	---	---
Child (20 kg)[b]	25	---	---
Mouse	3	12.3	0.08
Hamster	5	7.4	0.13
Rat	6	6.2	0.16
Ferret	7	5.3	0.19
Guinea pig	8	4.6	0.22
Rabbit	12	3.1	0.32
Dog	20	1.8	0.54
Primates:			
Monkeys[c]	12	3.1	0.32
Marmoset	6	6.2	0.16
Squirrel monkey	7	5.3	0.19
Baboon	20	1.8	0.54
Micro-pig	27	1.4	0.73
Mini-pig	35	1.1	0.95

[a] Assumes 60 kg human. For species not listed or for weights outside the standard ranges, HED can be calculated from the following formula:

HED = animal dose in mg/kg x (animal weight in kg/human weight in kg)$^{0.33}$.

[b] This k_m value is provided for reference only since healthy children will rarely be volunteers for phase 1 trials.

[c] For example, cynomolgus, rhesus, and stumptail.

The HED conversion table above also includes factors for converting between adult humans and children. In addition to differences in surface area, there are often related differences in metabolism and metabolic enzymes between the same species at different developmental stages: embryos, fetuses, newborns, juveniles, and adults. For example, it was recognized in the 1970s, that a cytochrome P-450 enzyme produced a chemically reactive intermediate from APAP, but it would take several years for NAPQI to be postulated and identified.[85] These scientists also reasoned that because the livers in young animals are immature, and expressed fewer cytochrome P-450 enzymes, young animals were expected to be relatively resistant or less vulnerable to APAP hepatotoxicity. This hypothesis was tested and supported in both mice and rats (Table 2).

[85] Reviewed in Hinson. Reactive metabolites of phenacetin and acetaminophen: a review. Environ Health Perspect. 1983 Mar;49:71-9. doi: 10.1289/ehp.834971. PMID: 6339229; PMCID: PMC1569121.

Age	24 Hour LD_{50} Mouse	Rat
7 days	3850(3500-4235)	2350(1942-2840)
14 days	2100(1590-2772)	1200(930-1606)
21 days	2150(1653-2795)	1700(1214-2180)
28 days	1500(1000-1960)	1500(1230-1900)
Adults	800(320-1760)	1580(1285-1900)

LD_{50} in mg/kg 24 hours after ip administration

( )=95% confidence limits

Table 2. Acetaminophen (APAP) Induced Lethality in Mice and Rats by Age. Higher dosages of APAP were required to produce a lethal dose (LD50) in younger mice or rats.[86]

Based on these LD50 data from adult mice and rats, the HED is estimated at 65mg/Kg (800mg/12.3) and 255 mg/Kg (1580mg/6.2), and 313mg/Kg and 379mg/Kg from 7-day old animals, respectively. In all cases, these calculations put the calculated HEDs (65-379mg/Kg) within the range of potential toxicity in humans (≥ 150 mg/kg).

The HED concept is important because dosages from animal studies should be converted based on body surface area and metabolism prior to comparing with human dosages, and these converted results allow researchers to appropriately compare results between humans and the various animals used in experimental studies.

As dosing humans with hepatoxic dosages is unethical, animal studies have been used to examine hepatic necrosis caused by various APAP dosages. For example, a dose of 400 or 600mg/Kg IP in Long Evans male rats resulted in liver necrosis in a minority (20-45%) of animals with no deaths reported, but at 800mg/Kg a majority developed necrosis and 10% died. This LD10 dosage produces a calculated HED of 129mg/Kg (800mg/6.2). At 1000mg/Kg (HED 161mg/Kg), all animals showed variable amounts of liver necrosis and mortality was 33%.[87] This range of toxicity in rats from 800mg-1000mg/Kg (LD10-LD30) produces HEDs from 129-161mg/Kg, and this encompasses the referenced potential for a human toxic dose reported in the Merck Manual, ≥ 150 mg/kg (about 7.5 g in adults). For clarification, a 7.5-gram overdose for a reference human of 60Kg (132lbs) produces a dose of 125mg/Kg. Using these estimates, the reported rat toxicities can be used for rat-human dosage conversion and produce overlapping HEDs and estimated toxic human dosages for APAP.

[86] Mancini et al. Developmental susceptibility to acetaminophen toxicity. Res Commun Chem Pathol Pharmacol. 1980 Mar;27(3):603-6. PMID: 7384649.

[87] Price and Jollow . Increased resistance of diabetic rats to acetaminophen-induced hepatotoxicity. J Pharmacol Exp Ther. 1982 Mar;220(3):504-13. PMID: 7062262.